



INVESTOR IN PEOPLE

The Patent Office
 Concept House
 Cardiff Road
 Newport
 South Wales
 NP10 8QQ

REC'D 10 MAR 2005

WIPO PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

PRIORITY DOCUMENT
 SUBMITTED OR TRANSMITTED IN
 COMPLIANCE WITH
 RULE 17.1(a) OR (b)

Signed

Dated 28 January 2005



Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
PE
30 NOV 2004
RECEIVED BY FAX

The
Patent
Office

30NOV04 E945381-1 D02973
P01/7700/0.00-0426252.3 ACCOUNT CHA

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP9 1RH

1. Your reference
P104749GB

2. Patent application number
(The Patent Office will fill in this part)

30 NOV 2004

0426252.3

3. Full name, address and postcode of the or of each applicant (underline all surnames)

08072423002

Patents ADP number (if you know it)

Intercytex Limited
Innovation House
Oaks Business Park
Crewe Road, Manchester
M23 9QR

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

Wound healing composition

5. Name of your agent (if you have one)

Harrison Goddard Foote

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Orlando House
11c Compstall Road
Marple Bridge, Stockport SK6 5HH
United Kingdom

Patents ADP number (if you know it)

00014571002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number
(if you know it)

Date of filing
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

- a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or

Yes

- c) any named applicant is a corporate body.
See note (d))

Patents Form 1/77

0125556 30-Nov-04 04:49

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form.
Do not count copies of the same document

Continuation sheets of this form

Description 35

Claim(s) 6

Abstract -

Drawing(s) 9 *only 1/1*

10. If you are also filing any of the following, state how many against each item.

Priority documents -

Translations of priority documents -

Statement of inventorship and right to grant of a patent (Patents Form 7/77) -

Request for preliminary examination and search (Patents Form 9/77) 1

Request for substantive examination (Patents Form 10/77) -

Any other documents (please specify) 2

covering letter and fee sheet

11.

I/We request the grant of a patent on the basis of this application.

Signature *Harrison Goddard Foote*

Harrison Goddard Foote

Date

30/11/04

12. Name and daytime telephone number of person to contact in the United Kingdom

Punita Davies

0161 427 7005

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

- If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.
- Write your answers in capital letters using black ink or you may type them.
- If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- Once you have filled in the form you must remember to sign and date it.
- For details of the fee and ways to pay please contact the Patent Office.

Patents Form 1/77

DUPLICAT

WOUND HEALING COMPOSITION

Field of the invention

The invention concerns the delivery of live cells with the capacity to initiate and encourage the healing process, preferably in a biocompatible matrix, to a wound. The cells and compositions comprising them are useful for assisting the process of wound healing, particularly chronic open lesions that are slow or resistant to heal.

Background to the invention

Healing of open wounds extending through the germinal epithelium in otherwise healthy tissue takes place by the process classically described as 'second intention', which, following initial haemostasis, involves a well-ordered sequence of inflammation, cellular infiltration, angiogenesis, granulation and re-epithelialisation. Where the wound has destroyed the germinal layer of the epithelium, collagen deposition by infiltrating fibroblasts and re-epithelialisation results in a degree of scarring, with incomplete restoration of function in terms of the flexibility and elasticity of the original dermis and failure to regenerate auxiliary structures such as hair follicles and sweat glands. Various approaches of autologous skin-grafting have long been used to close open wounds, minimise the risk of opportunistic infection, accelerate healing and minimise scarring.

However, a number of factors may adversely affect the rate and extent of such wound healing, in particular, poor blood supply. Poorly perfused tissue, often associated with impaired venous return and varicose veins, peripheral vascular disease or diabetes, often fails to heal satisfactorily, resulting in chronic ulcers, although the details of the pathogenesis are still unclear.

Current methods of skin grafting have significant limitations, not least the requirement for a suitable donor site from which grafts can be taken, especially where wounds are extensive (for example, burns). In addition, grafts have a low success rate where wound healing is compromised.

Chronic leg ulcers are a significant and growing problem world-wide. The introduction of compression therapy in combination with moist wound dressings has been the standard therapeutic management. More recently, tissue-engineering solutions have become available. Research into regenerative medicine has shown that human cells have substantial potential to heal and regenerate damaged tissue especially when primed by an environment that closely mimics the natural physiological condition being treated.

Much of the research has focused on the production of so-called 'tissue equivalents', which aim to provide a temporary functional replacement for missing tissue and accelerate healing. Such tissue equivalents may be dermal equivalents or total skin equivalents, the aim being to provide effective coverage of the wound as quickly as possible. The development and production of tissue equivalents usually involves the isolation of replacement skin cells, which are expanded and seeded onto / into a supporting structure such as a three-dimensional bio-resorbable matrix, or within a gel-based scaffold.

A variety of materials have been used as acellular protein matrices for wound healing applications. These include synthetic polyesters (polyglycolic acid (PGA), polylactic acid (PLA), polyglactide (DermagraftTM, Smith & Nephew, described below), polydioxanone, polyhydroxyalkanoates and hyaluronic acid derivatives), hydrophilic polyurethanes (polyetherpolyester, polyethylene oxide and carboxymethylcellulose ethylene), and collagen-based scaffolds (cross-linked elastin collagen material (MatridermTM), cross-linked collagens manufactured from acid-soluble type I bovine collagen material (such as VitaphoreTM). An alternative approach is to use an acellular derivative of allogeneic human dermis, a natural dermal matrix from which cells have been removed (such as Alloderm[®], LifeCell Corporation). Some preparations use an organised, layered structure in order to more closely mimic the structure and function of the dermis. For instance, a preparation comprising an underlying layer of bovine collagen and shark glycosaminoglycans with an overlying layer of silicone is known (Integra[®], Integra LifeSciences

Corporation).

Other approaches have involved utilising fibrin sealants. Fibrin sealants (such as Tisseel™, Baxter; Beriplast™, Aventis; Quixil™, Omrix Biopharmaceuticals; Haemaseel™, Haemacure; and Crosseal™, Omrix) have been available for some time. All of these commercially available fibrin sealants are derived from cryoprecipitate from pooled plasma from virally screened allogeneic donors.

Such fibrin products rely on the natural polymerisation process that occurs during the physiological blood clotting cascade, in which a monomeric fibrin precursor, fibrinogen, is acted on by activated thrombin with the resultant production of polymeric fibrin. Fibrin forms the protein scaffold component of blood clots, to which platelets adhere.

More recently, fibrin has been recognised as a convenient and clinically acceptable cell carrier to be used in tissue engineering applications. Commercially available products that utilise fibrin sealants for cell delivery include Bioseed™ (Biotissue technologies), although several research groups make reference to the use of fibrin sealants for cell delivery purposes results of which have shown some promise in burns (Brown *et al*, 1993, *Am J Pathol* 142: 273–283; Neldert *et al* 2001 Proceedings of the ASME Bioengineering Conference, Eds, Kamm *et al*, Vol 50: 215–216; Tuan *et al*, 1996, *Exp Cell Res* 223: 127–134; and US Patent application 2003/01654482).

Exogenously applied dermal cells have been consistently shown to have beneficial effects on wound healing including shorter time to complete healing (Falanga and Sabolinski, 1999, *Wound Repair Regen* 7: 210–207); delivery of active growth factors to the wound (Naughton *et al*, 1997, *Artif Organs* 21: 1203–1210); reduced potential for lesion recurrence (Gentzkow *et al*, 1996, *Diabetes Care* 19: 350–354; and reduced pain (Muhart *et al*, 1999, *Arch Dermatol* 135: 913–918).

As part of the normal healing response resident fibroblasts are required to undergo a series of phenotypic changes, migrating to the wound site, then proliferating, then synthesising and secreting extracellular matrix molecules. *In vivo*, a least a proportion then switch to a myofibroblastic phenotype in order to facilitate wound contraction. *In vitro*, a series of phenotypically distinguishable mitotic and post-mitotic fibroblast populations have been described (Bayreuther *et al*, 1988, Proc Natl Acad Sci USA 85: 5112–5116). This differentiation pathway appears to be controlled, at least in part, by interactions between fibroblasts and extracellular matrix (ECM) proteins present at the wound site. Growth factors and cytokines undoubtedly also exert an important influence, although their effects too, appear to be modulated by fibroblast exposure to particular ECM proteins. Among the ECM proteins that appear to have an important role in fibroblast differentiation are fibrinogen and fibrin. Fibroblasts specifically interact with fibrin and fibrinogen RGD motifs through $\alpha_v\beta_3$ integrin receptors although the cellular response is complex and modulated by other factors. *In vitro* studies of the effect of fibrin glue on human periodontal ligament fibroblasts have suggested that fibrin appeared to slightly inhibit fibroblast proliferation. The presence of a fibrin matrix has also been reported to increase the synthesis of collagen by entrapped fibroblasts (Neidert *et al*, 2001) The interaction between fibroblasts and ECM, in particular fibrin, and its influence on the activation and/or differentiation of fibroblasts is clearly of relevance when considering wound healing compositions comprising fibrin and live fibroblasts and attempting to optimise their therapeutic effects.

Fibroblasts also have a role in the remodelling of fibrin clots. As new extracellular matrix proteins such as collagen type I and III, fibronectin and vitronectin are laid down, the fibrin matrix is broken down, predominantly by the activation of the plasma-derived enzyme plasmin. This is regulated by the activation (or inhibition) of its proenzyme, plasminogen, by a variety of plasminogen activators and inhibitors. *In vivo*, a number of infiltrating cells, such as neutrophils and macrophages, secrete urokinase-type plasminogen activator (uPA), whilst endothelial cells are largely responsible for producing tissue plasminogen activator (tPA). However, it also clear that fibroblasts also

secrete both uPA and plasminogen activator inhibitors, such as plasminogen activator inhibitor-1 (PAI-1). The balance between these antagonistic mediators is crucial in controlling fibrin remodelling and scar formation. It is also clear that their expression is developmentally regulated, as well as being controlled by extracellular matrix components and local growth factors.

To facilitate movement through the cross-linked fibrin clot and a tight meshwork of extracellular matrix, a variety of fibroblast- and serum-derived enzymes cleave a path for migration. These include interstitial collagenase (matrix metalloproteinase-1, MMP-1), gelatinase (matrix metalloproteinase-2, MMP-2), stromelysin (matrix metalloproteinase-3, MMP-3) and the plasminogen activators. Additionally, chemotactic factors, such as TGF- β and PDGF may also upregulate their production and secretion.

Once migrating fibroblasts reached the wound, they gradually become secretory, whose major function is protein synthesis. The previously retracted endoplasmic reticulum and Golgi apparatus becomes dispersed throughout the cytoplasm and a loose matrix is produced, which is mainly composed of fibronectin and type III collagen. Ultimately, this profibrotic phenotype takes over, which is characterised by an abundance of rough endoplasmic reticulum and golgi apparatus, secreting newly synthesised collagen in response to highly expressed TGF- β . Notwithstanding, TGF- β fails to upregulate further collagen deposition, once a matrix has been deposited. It is also thought that IL-4 released by mast cells induces a modest increase in types I and III collagen together with fibronectin. Mast cells furthermore produce tryptase (a serine esterase) in abundance, which has been shown to upregulate fibroblast proliferation.

The stimuli responsible for fibroblast proliferation and matrix synthesis (TGF- α , TGF- β and PDGF) have been extensively investigated *in vitro* (Derynck R (1988) *Cell*, 54: 593-595; Ross RR and Raines EW (1990), Platelet-derived growth factor and cell proliferation, in: *Growth Factors: From genes to clinical applications* (VR Sara et al. eds.) pp. 193-199, Raven Press, New York; Sporn MB and Roberts AM (1992) *J Cell Biol*, 119:1017-102) and their action

was confirmed by *in vivo* manipulation of wounds as well (Sprugel KH, McPherson JM, Clowes AW, Ross R (1987) *Am J Pathol*, 129: 601-613; Pierce GF, Mustoe TA, Altmann B, Deuel TF and Thomas A (1991), Role of platelet-derived growth factor in wound healing, *J Cell Biochem*, 45:319-326). γ -interferon on the other hand was demonstrated to have a negative effect on the mitogenic and synthetic potential of fibroblasts *in vitro* and *in vivo* (Duncan MR and Berman B (1985) *J Exp Med*, 162: 516-527; Granstein RD, Murphy GF, Margolis RJ, Byrne MH and Amento EP (1987) *J Clin Invest*, 79: 1254-1258). In addition, the collagen matrix itself can suppress these activities (Grinnell, 1994; Clark *et al.*, 1995), whilst fibrin or fibronectin matrix has little or no suppressive effect (Clark *et al.*, 1995).

Interestingly, many fibroblasts undergo apoptosis (programmed cell death) in day-10 healing wounds, thereby marking the transition from a fibroblast-rich granulation tissue to a scar tissue with reduced cell density.

Known combinations of protein matrices and live cells for wound healing applications may be summarised as follows.

A preparation comprising cryo-preserved primary human foreskin fibroblasts seeded onto a bioabsorbable glycolic-lactic acid polyester (polyglactide) scaffold (DermagraftTM, Smith & Nephew) is known for the treatment of ulcers (Naughton *et al.*, 1997, US patent nos. 4,963,489,). The fibroblasts are allowed to proliferate in the scaffold, secreting extracellular matrix proteins and growth factors and cytokines. The mature preparation is packaged in 10% dimethylsulphoxide and bovine serum as a cryoprotectant to allow storage of the product by freezing prior to use. The disadvantages of this approach include difficulty in manipulating the product during application to the wound, and the necessity of storing and transporting the product at very low temperatures (-70°C) and use of careful thawing procedures in order to ensure viability of the cells (WO 87/06120).

Various combinations of collagen-based matrices and living cells are known. Apligraf[®] (Organogenesis Inc), is a bilayered structure comprising a lower

('dermal') layer of a bovine collagen scaffold supporting living human fibroblasts and an upper ('epidermal') layer comprising human keratinocytes on a collagen scaffold (Falanga and Sabolinski, 1999, WO 99/63051). The preparation is supplied as a circular disk approximately 75 mm in diameter and 0.75 mm thick on an inert polycarbonate membrane. It is packaged individually for use and has 5-day shelf life. It is maintained in an agarose rich nutrient with a 10% CO₂/ air atmosphere and is shipped and stored at room temperature (20°C to 31°C; 68°F to 88°F). The removal of the product from the storage dish and polycarbonate membrane involves teasing away the edge of the Apligraf using sterile forceps. Problems associated with this include excessive folding which can make accurate, close application of the preparation to the wound difficult and time consuming.

A similar product (Orcel™, Ortec International Inc) is described in US patent 6,039,760. This is also a bilayered structure of bovine collagen with fibroblasts and keratinocytes. The preparation is packaged between 2 non-adherent pieces of mesh, which are differently coloured to distinguish between sides. The device is then packaged in a plastic tray containing media to maintain cell viability during storage and shipping, which is further packaged into pouches with chill packs to maintain a temperature of 11-19°C for 72 hours.

Other examples of tissue equivalents that attempted to reproduce a dermis-like arrangement of fibroblasts in a protein matrix supporting an overlying layer of keratinocytes are described in Meana *et al* (1998, Burns 24: 621-630).

Rama *et al* (2001, Transplantation 72: 1478-1485) describe a method of culturing autologous limbal stem cells on a fibrin gel substrate for grafting to the contralateral cornea.

US Patent application 20030165482 discloses a wound healing preparation (Allox™, Modex Therapeutiques SA) comprising growth-arrested allogeneic human fibroblasts applied to a wound in a viscous paste of fibrinogen

(Tisseel®) to which thrombin has been added, so that fibrinogen cleavage and fibrin polymerisation occur *in situ*. Alternatively, the separate liquid components are sprayed onto the wound, to set *in situ*, on mixing.

Summary of the invention

The invention provides an approach to treatment of chronic wounds based, not on providing an immediately functional tissue-equivalent, but on delivering cells with the potential to accelerate the healing process, optionally with a supportive, biocompatible protein matrix. Although developing a cultured dermal tissue equivalent comprising fibroblasts, extracellular matrix and overlying keratinocytes organised in to functional and anatomically relevant structures remains a worthwhile goal, so far this has proven elusive. However, for many situations, the present invention shows that such an approach may be unnecessarily complex and that a simpler solution, that of providing cells at the appropriate stage of maturity and exhibiting a particular phenotype, preferably in a wound-healing composition for rapid, convenient and accurate application to wounds, is surprisingly effective. Optimally the cells must be in a synthetic phase of maturity, rather than a proliferative or senescent phase. Proliferation maybe useful to increase cell numbers, but delays the important synthesis of extracellular matrix proteins such as collagen types I and III, fibronectin and vitronectin. Cells that have become senescent do not contribute to wound healing and so serve little purpose as such a therapeutic. An important aspect of the invention, therefore, is provision and use of cells with a "wound-healing phenotype" (a phenotype that is either actively synthetic, or that can rapidly mature to such a phenotype), to encourage immediate wound healing, and the delivery of such cells to a wound, preferably in a manner which is consistent with the maintenance of such a phenotype.

Another important factor is the rate of fibrinolysis occurring within the composition. As described above, fibrinolysis is a normal part of the wound healing process, by which the fibrin matrix is gradually replaced by other extracellular matrix proteins. If, however, fibrinolysis occurs too early or too

rapidly, the wound healing composition is broken down before useful collagen deposition has occurred. Expression of pro-fibrinolytic factors such as urokinase-type plasminogen activator is developmentally regulated and so the phenotype of the cells included in the composition is of importance if premature fibrinolysis is to be avoided.

The cell phenotype is therefore critical to optimal wound healing. Preferably early gene expression will include elevated collagen gene expression and other genes involved in the early exposure of fibroblasts to a wound environment.

The present inventors have used the three different methods described below to identify a gene expression profile of an appropriate early phenotype:

1. Quantitative PCR (TaqMan) is used to measure the amounts of particular genes being expressed by fibroblasts. The genes screened included: Gas6 (growth arrest-specific 6, also known as AXSF or AXLLG), colla1 (collagen type I α 1), col3a 1 (collagen type III α 1) col6a1 (collagen type VI α 1), PAI-1 (plasminogen activator inhibitor type-1), INSIG-1 (insulin-induced gene 1), PLAU (plasminogen activator, urinary or urokinase), COX-2 (cyclo-oxygenase-2), PDGF (platelet-derived growth factor), Vim SMA (smooth muscle actin), ApoD (Apolipoprotein D), MMP-2 (matrix metalloprotease 2, gelatinase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and RPL32 (ribosomal protein L32). Other informative genes include those encoding cytokines, metabolic genes, cytoskeletal genes, cell surface molecules and cell signalling molecules.
2. Differential display is a PCR-based method using non-specific primers, which produces a banding pattern when run on a gel that is unique to the sample of interest. This results in a "barcode" type pattern of gene expression. The advantage of this process is that it is fairly quick to perform and produces an easily recognisable pattern that can be analysed

without numerical manipulation, or knowledge of the actual genes involved.

3. Microarray is a powerful technique for looking at global gene expression patterns. The advantage of this technique is that it is possible to assess the expression of a large sample size of ~20,000 genes expressed by human cells. Therefore it is likely to capture all relevant genes.

It has been found that taking passaged human dermal fibroblasts, seeding them in a protein matrix and then incubating them for 0-96 hours results in a phenotype that is particularly beneficial for use in wound healing applications. It has been observed that such cells are predominantly in a proliferative phase in culture (encouraged by low density seeding, avoiding contact inhibition). Under normal culture conditions, maturation to a wound-healing phenotype typically takes 2-3 days. However, incubation of fibroblasts in a suitable environment such as a matrix or wound shortens this maturation process, so that before 96 hours the cells are entering a wound-healing phenotype. Moreover, once they have reached this phase, they can preferably conveniently be stored at approximately 4°C for up to 28 days, and certainly 7-14 days before use without significant loss of viability or change of phenotype. This has significant practical advantages in that it provides not only an efficacious product comprising cells that are optimally suited for secretion of extracellular matrix with minimal inappropriate fibrinolysis, but also gives a relatively long shelf-life under commonly available standard refrigeration conditions. The ability to ship such products at approximately 4°C also considerably simplifies transportation. Maintaining a cold chain at 2°-8°C is considerably simpler and cheaper than shipping at -70°C, as is commonly required for live cells.

Thus present invention provides a wound healing composition comprising living mammalian cells having a wound healing phenotype, wherein the cells:

- (a) have been incubated in a protein rich environment for approximately 0-96 hours; and/or

- (b) exhibit 100 – 2000 fold increase in expression of ApoD, 13000 – 100000 fold increase in expression of MMP2, 800 to 1800 fold increase in expression of Col3a1, and/(or) 1600 to 2500 fold increase in expression of SMA, as compared to the expression of RPL32; and/or
- (c) have a banding pattern of PCR products resulting from differential display, similar to that shown in Figure 4 or Figure 5.

Through the above mentioned analysis of gene expression, it has been observed that the four genes in (b) are expressed from days 1 to 14, and are indicative of a cellular phenotype which is effective at accelerating or assisting wound healing. The expression of these genes has been observed to be independent of temperature, and thus this wound healing profile of the cells is maintained throughout storage and shipping which may take place during the above time frame.

The present inventors have obtained cells having a wound healing phenotype by incubating cells in fibrin. This phenotype, which is described above, is believed to represent the optimal phenotype for accelerating or assisting wound healing. Whilst incubating in fibrin is a preferred way of obtaining such cells, it is envisaged that cells of this particular phenotype may be obtained by placing them in other suitable environments. Suitable environments include those rich in fibrin, such as a matrix (herein referred to as a maturation matrix) or a wound. Preferably, the cells are incubated in this environment for a maximum of 0 – 96 hours, 0 – 48 hours, 0 – 25 hours and most preferably 16 – 24 hours, during which they reach a wound healing phenotype.

The level of gene expression is measured in terms of fold increase compared to the expression level of the housekeeping gene RPL32. A housekeeping gene is one whose expression is largely independent of intrinsic and extrinsic factors that might influence gene expression and thus serves as a point of standardisation for genes whose expression may vary according to such factors. Thus, comparison of gene expression to a housekeeping gene

provides an indication of gene expression level which is independent of factor such as starting conditions, amounts of RNA, or amounts of product. The expression levels of both RPL32 and the genes of interest can be measured using standard methodology, available to the skilled person, such as PCT, quantitative PCT (Taqman) and Northern blot.

The preferred fold change in expression levels of the genes of interest as compared to RPL32 are as follows:

ApoD - 2 to 48,000, preferably 100 to 2,000;

MMP2 - 2,000 to 1,600,000, preferably 13,000 to 100,000

Col3a1 - 20 to 44,000, preferably 800 to 1,900

SMA - 20 to 150,000, preferably 1,600 to 8,600, most preferably 1,600 to 2,500

In addition, cells having the desired phenotype have been observed to express DD5 (X-ray repair complementing defective in hamster 1) between 0 to 500, preferably 13 to 160; DD10 (gi10437022) in the range 0 to 210, preferably 3 to 15; and/or GB1 (gi24810897) in the range 0 to 33, preferably 0 to 5. Thus, the present invention provides living mammalian cells having the specified amounts of DD5, DD10 and/or GB1 in addition to the gene products mentioned above.

Preferably, the cells also express GB5 (Ribosomal protein S24) in the range 1,000 to 120,000, preferably 11,000 to 53,000, most preferably 100,000; DD12 (Ribosomal protein S8) in the range 120 to 36,000, preferably 1,000 to 30,000; and DD2 (gi 14626 7369) in the range 0 to 750,000, preferably 0 to 4 during days 1 to 14 and 0 to 36,000 at 4°C.

Additional markers of cells having a wound healing phenotype have been identified, which show temperature dependence and therefore whose expression may vary between culturing and storage/shipping conditions. Specifically, GB5 in the range of 1,000 - 120,000, or more preferably 1,000 - 53,000; Gas 6 in the range of 0.01 - 8 or more preferably 0.1 - 4 at days 1 -

5 in the cold (e.g. 4°C); GAPDH in the range 140 – 12,000,000 or more preferably 10,000 – 38,000 in the cold; DD2 (gi7022020) in the range of 0.01 – 750,000 more preferably 0.01 – 4 at days 1 to 14 or 0.01 – 136,000 in the cold; DD12 (ribosomal protein S8) in the range 120 – 136,000 or more preferably 1,000 – 30,000; PLAUI the range 9 – 9,400 or more preferably 130 – 760; and/or Vimentin in the ranges 13,000 – 50,000,000 or more preferably 28,000 – 2,100,000.

The ranges are detailed in table 5.

The PCR products resulting from differential display in (c) are believed to be:-

227bp – DD2

347bp – DD4

333bp – DD5

128bp – DD10

478bp – DD12

157bp – DD13

396bp – GB1

398bp – GB5

Thus, wound healing cells expressing nucleic acids encoding the above genes may fall within the scope of the invention even if the PCR products differ in size from those indicated in the figures. The cells having the desired phenotype will typically express one or more of these genes under particular conditions or at a particular age, as shown in Table 4.

For delivery, the cells having a wound healing phenotype are provided in a wound healing composition, comprising a suitable medium or a biocompatible matrix (a delivery matrix). The matrix may be a clottable or gelling substance, and preferably is protein based. Preferred biocompatible matrix substances include agar, alginate, PVA, collagen, fibronectin and vitronectin. A most preferred matrix substance is fibrin, preferably fibrin gel. Preferably, the matrix is sterile and non-pyrogenic.

In a preferred embodiment, the cells are cast in the matrix prior to topical application. Thus, the present invention also provides a wound healing composition comprises living mammalian cells within a sterile, non-pyrogenic matrix characterised in that said matrix is pre-cast before topical application.

Most preferably, the cells are cast in the matrix prior to maturation to a wound healing phenotype.

The composition preferably takes the form of a solid or semi-solid topical medicament, such as a gel. Where the composition is sufficiently solid, it may ~~be provided in any suitable shape and size, to suit the lesions it is design to~~ be used with. Preferably it is substantially disk-shaped. Preferably, such compositions are less than 8mm thick, and most preferably less than 5mm thick.

Preferred cells of the present invention include fibroblasts, keratinocytes, stratum germinativum cells, and combinations or admixtures of such cells. Preferred cells are fibroblasts, preferably dermal fibroblasts. The cells may be isolated from any suitable mammalian source, and preferably are human. The cells are preferably allogeneic. As required for manufacture, cells are thawed, recovered, expanded for approximately 7 days in culture or until they reach confluence, and resuspended in appropriate volumes and densities as required. Although early passage cells are strongly preferred, the exact passage number is not critical. Preferably it less than 20, more preferably it is less than 15, most preferably less than 10, preferably 7. Once defrosted for use in the present invention, the cells may be matured as described above.

For the purposes of the present invention, day 0 is the day on which the cells begin maturation and they will reach a wound healing phenotype up to 4 days, or 96 hours, after day 0.

In the solid or semi-solid forms, the composition contains approximately 450 to 2500 cells per mm^2 , preferably 500 to 1500 cells per mm^2 , and more preferably 450 to 550 cells per mm^2 . Alternatively, such a composition may

contain approximately 750 to 2000 cells per mm^2 , preferably 900 to 1700 cells per mm^2 , most preferably 1450 to 1550 cells per mm^2 as measured per unit area. Lower cell densities result in poor cell viability and higher result in inhibition of extracellular matrix protein synthesis and progression to a senescent cell phenotype. Within this range of cell densities, particular products have been developed using approximately 500 cells per mm^2 and approximately 1500 cells per mm^2 . For non-solid forms of the composition, the number of cells will be sufficient to cover the above ranges per mm^2 as defined above.

Where a matrix is used, it preferably has a protein concentration in the range 5-20mg ml^{-1} , preferably 7-12 ml^{-1} more preferably 3-12mg ml^{-1} and most preferably 3-5 ml^{-1} .

The composition has a shelf-life of at least 7-11 days, and preferably up to 28 days, more preferably 21 days, at 2°-8°C.

Also provided is method of manufacturing a wound healing composition, preferably as described above, comprising: suspending living mammalian cells in a solution comprising a protein monomer capable of polymerisation into an insoluble matrix, adding an agent capable of promoting such polymerisation and allowing polymerisation to occur in a mould such that the solid polymerised composition may be removed and packaged ready for topical administration to a patient. The cells are preferably as above.

The cells may have a wound healing phenotype as described above prior to being suspended in the protein monomer, or may adopt such a phenotype within 0-96 hrs after suspension.

Preferably, said monomer is fibrinogen and said agent capable of promoting polymerisation include thrombin, vitamin k dependent clotting factors, venom serine proteases (eg, Crotalax, Batroxobin, Gabonase, Okinaxobin, Reptilase, Calobin and Fibrozyne) and others with thombin -like fibrinogen cleaving activity.

Preferably, the composition of the invention may additionally comprise a protease inhibitor, suitable for preventing breakdown of the matrix being used in the composition. Preferably, the inhibitor is a serine protease inhibitor, most preferably one selected from the list consisting of aprotinin, ϵ -aminocaproic acid, tranexamic acid. Preferably, especially where the concentration of protein is in the range $7\text{--}12\text{mg ml}^{-1}$, the protease inhibitor is aprotinin. Alternatively, especially where the concentration of protein is in the range $3\text{--}5\text{mg ml}^{-1}$, the protease inhibitor is tranexamic acid.

The present invention also provides the cells and compositions as herein described for use as a medicament, preferably for the treatment of a skin lesion.

In addition, the invention provides the use of cells as herein described in the manufacture of a composition to treat a skin lesion. Preferably, there is provided the use of the cells of the invention and a suitable medium or biocompatible matrix in the manufacture of a wound healing composition for the treatment of a skin lesion. The composition is preferably manufactured as described herein.

In another aspect the invention provides a method of treating a patient suffering from a skin lesion or other appropriate condition, comprising the topical application of a wound healing composition as herein described.

The method of treatment may comprise applying a solid or semi-solid topical composition by means of flexible pouch composed of two sheets of impermeable flexible material sealed to provide a means of containment and in which the composition is preferentially adherent to the internal surface contributed by one of said sheets; comprising opening the pouch by parting the sealed sheets and manipulating the composition whilst adherent to one of the sheets and applying the composition to the surface to be treated without any requirement for the composition to be directly touched by any other means.

In the present invention, the skin lesion may be an ulcer or a burn, preferably a venous ulcer, diabetic ulcer, pressure sore, burn, or iatrogenic grafting wound.

In another aspect, the invention provides a means of storing and transporting a medicament comprising living mammalian cells at 2°-8°C.

In a further aspect, the invention provides a package for a solid, or semi-solid, sterile, topical composition comprising a flexible pouch consisting of two sheets of impermeable flexible material peripherally sealed to provide a means of containment, said pouch comprising one internal surface to which said composition is preferentially adherent but to which the level of adhesion is less than that between the composition and the bodily surface to be treated, such that the pouch may be opened by parting said sheets and the composition conveniently manipulated and directly to the surface to be treated without any requirement for the medicament to be directly touched by any other means before application. Preferably, the composition and cells are as described herein.

It is clear that such a package provides a convenient means of storage, delivery and application of any form of solid or, especially, semi-solid, materials, especially those intended for topical application to bodily surfaces. Preferably such materials are of a semi-solid or gel nature, such that physical manipulation is difficult. The preferential adherence of the material to an element of the packaging, with the ease of transfer thereafter to the skin or other bodily surface, provides a considerable advantage. In particular, such materials may be cut to the required size before application to the intended area. In the case of wound healing compositions as herein described, this is a particular advantage.

In a preferred embodiment, the package comprises metal foil, laminated or metalised plastic. In one preferred embodiment it comprises a transparent area allowing visual inspection of its contents.

Preferably, the internal surfaces of the package and its contents are sterile.

In a preferred embodiment one internal surface of the pouch is modified to increase the adherence of the composition thereto. In one embodiment this comprises application of a coating to one internal surface. Preferably the coating is selected from the list consisting of: a polymer, a thermoplastic, a thermo-setting plastic, a protein, an amino acid, a carbohydrate.

Alternatively, one internal surface is modified by roughening to increase the adherence of the composition thereto. As used herein, 'roughening' includes any physical modification of the surface intended to improve adherence, such as embossing, scratching, abrading or scuffing, or chemical roughening by means of etching, erosion, acid or alkali treatment. Other means of modifying the surface energy properties of the surface in order to improve or modulate the degree of adherence of the solid or semi-solid product are disclosed. Such means include coating one internal surface of the pouch. Preferably such a coating is selected from the list consisting of a polymer, thermoplastic, thermo-setting plastic, protein, amino acid or carbohydrate.

In one particularly preferred embodiment, the internal surface is modified by means of a discontinuous coating, in the form of raised areas or dots, having the effect of providing a roughened surface.

Also provided is a method of packaging a sterile, solid or semi-solid topical composition as described herein comprising: placing said composition in a sterile flexible pouch consisting of two sheets of impermeable flexible material incompletely peripherally sealed to provide a means of partial containment, said pouch comprising one internal surface to which said composition is preferentially adherent but to which the level of adhesion is less than that between the composition and the bodily surface to be treated; and completely peripherally sealing said pouch to provide a means of containment. Preferably, the packaging is as described herein.

One internal surface of the pouch is preferably modified to increase the adherence of the composition thereto, as herein described.

Detailed description of the invention

The invention will now be described in detail with reference to the figures, wherein:

Figure 1 summarises the process of manufacturing the wound healing composition.

Figure 2 shows the packaging, manipulation and application of the wound healing composition. **A:** shows the set gel preferentially adhering to the modified internal surface of one of the two metalised plastic sheets of which the pouch is comprised. **B:** shows the use of one of the sheets of the package to apply the gel of wound healing composition to skin. Note that sheet may used to support the gel while both are cut to the appropriate shape and size. **C:** shows the wound healing composition in place.

Figure 3 is a diagrammatic representation of the differential display process.

Figure 4 shows an example of a "bar code" as revealed by differential display of polyA cDNA products between samples cast into bovine collagen (lanes 1,2,7,8,13,14,19,20), onto tissue culture plastic in no exogenous matrix (lanes 3,4, 9,10,15,16,21,22) or into fibrin (lanes 5,6,11,12,17,18,23,24) and RNA sampled from each matrix/condition on 1 (lanes 1-6), 5 (lanes 7-12), 14 (lanes 13-18), or 21 (lanes 19-24) days after casting.

Figure 5 shows a "bar code" of comparative of gene expression, as revealed by differential display, of cells in fibrin 1 (lanes 1 and 2), 5 (lanes 3 and 4), 14 (lanes 5 and 6), and 21 (lanes 7 and 8) days after casting. Arrows indicate specific PCR products that increase with increasing time from casting.

Figure 6 shows a "bar code" comparison of gene expression, as revealed by differential display, of human dermal fibroblast cells cast onto tissue culture plastic 1 (lanes 1 and 2), 5 (lanes 3 and 4), 14 (lanes 5 and 6), and 21 (lanes 7 and 8) days after casting. Arrows indicate specific PCR products that increase with increasing time from casting.

Figure 7 shows a graph comparing expression of Apolipoprotein D (ApoD) in HDFs cast in fibrin, collagen and onto tissue-culture appropriate plastic and stored at 4°C (sample 1) or at 37°C for 1 (sample 2), 5 (sample 3), 14 (sample 4), or 21 (sample 5) days. The shaded area on the graph represents the range of expression specific to the profile of young cells and the profile for storage/shipping conditions of the product of the invention.

Figure 8 as Figure 7 for Matrix Metalloproteinase 2 (MMP2) gene.

Figure 9 as Figure 7 for collagen 3A1 (col3A1) gene.

The process of manufacturing a preferred composition of the invention is summarised in Figure 1. Alternative components or methods may be used in place of those described below, as suggested above. In principle, the composition comprises two components, which are cast together. The first component comprises a solution of fibrinogen together with one or more protease inhibitors to prevent unwanted proteolysis by protease contaminants and premature matrix breakdown by cells during storage. In particular, contaminants may include the naturally fibrinolytic enzyme plasmin, or its precursor plasminogen. Serine protease inhibitors such as aprotinin, ϵ -aminocaproic acid, or its analogue tranexamic acid, are frequently used in order to inhibit plasmin or prevent its activation. Added to this fibrinogen solution is a suspension of living cells in a suitable medium or buffer solution. The second component comprises a solution of thrombin (an enzyme that naturally acts upon fibrinogen), calcium ions (a required cofactor), and a medium suitable for the culture of living cells. A further clotting factor, Factor XIII, is also activated by thrombin in the presence of calcium ions. Activated Factor XIII promotes polymerisation of monomeric fibrin (cleaved from

fibrinogen by thrombin) into a three-dimensional protein insoluble scaffold. In order to cast the gel, these two components are combined and poured into a pre-coated suitable mould whilst still liquid. Although commonly circular, clearly the gels may be cast into any shape. For some applications, other shapes may be more suitable. In particular, essentially rectangular or elliptical gels may be more convenient for larger wounds.

Enzymatic cleavage of fibrinogen into fibrin monomers and polymerisation of these monomers results in setting of the liquid into a semi-solid gel in which living cells are suspended. For many applications, this gel is then maintained for a period of about 24 hours under suitable conditions for cell growth, division and secretion of extracellular matrix proteins, and other proteins such as growth factors. Following maturation, the cast gels are removed from the casting moulds and placed directly into sterile packages. A small amount of medium is added to each package to maintain the product during storage and shipping, and the packages are sealed. During storage and shipping the packages are maintained at a temperature of 2°-8°C.

The advantages of such a product over the currently available alternatives include the following:

The use of a protein sealant as a scaffold allows convenient topical delivery of cells to the wound. The pre-cast gel allows convenient and accurate application of regenerative cells to the wound surface with control of the distribution and density of cells applied. Manufacture and shipping of other tissue equivalents takes approximately 3 weeks for the matrix alone, whereas the product of the invention may be manufactured in 10 days, or even as little as 2 days if sufficient growing cells are available. These factors combine to give cost advantages, so that manufacture and production is more cost effective than other commercially available products.

As described below, the product of the invention also features a unique flat pack system (adhesive backing) ensuring maintenance of product during shipping and "ease of use" of final product. The precast gels can be shipped

and stored for up to 28 days at 2-8°C, whereas other available products are either frozen or shipped at room temperature.

Example 1 High protein concentration product ('Protoderm 500' and '1500')

One embodiment is designed to optimise both rapid manufacturing of the product and rapid wound healing by containing cells and protein components at relatively high concentrations.

Matrix

In this embodiment the matrix protein is fibrin, derived from a commercial fibrinogen product, Tisseel® (Baxter). When reconstituted this provides a convenient two component system to which cells may be added. The key components of the matrix are summarised in Table 1. It should be noted that Tisseel® also contains Factor XIII, as well as plasmafibronectin and plasminogen.

Table 1

| Component | Final concentration in cellularised scaffolds |
|-----------------------------|---|
| Matrix protein (fibrinogen) | 7.5 - 11.5 mg/ml |
| Aprotinin | 300 K IU/ml |
| Thrombin | 25 IU/ml |
| Calcium chloride | 4 mM |

As will be clear to one of appropriate skill in the art, the exact concentrations of these components are not critical. Fibrinogen may be used in concentrations of the approximate range 7-20mg ml⁻¹ for this application, thrombin in the range 5-50 IU/ml (in fact, trace levels of contaminating thrombin will eventually lead to fibrin formation and gel setting without additional thrombin, but this is inconvenient and unpredictable), and calcium

chloride in the range 2–20mM. Aprotinin is used to prevent unwanted fibrinolysis but, again, the exact concentration is not critical.

Cells

Human dermal fibroblasts were obtained by culture of cells derived from neonatal foreskin tissue. Under GMP conditions, fibroblastic cells were isolated by collagenase digestion and expanded by culture and serial passage according to routine laboratory practice to establish a master cell bank (MCB). The MCB was screened against a panel of human and animal-derived viruses, bacteria, mycoplasma and fungi, and for tumorigenicity by a GLP-accredited facility and determined to be free of contamination. Several working cell banks (WCB) were then established for manufacture of the product, rescreened and stocks of cells frozen according to standard procedures.

It is also envisaged that for various patient-specific applications, autologous fibroblasts or other cells obtained from biopsies may be cultured and expanded for use.

The cells are suspended in Liebowitz L-15 cell culture medium buffered and supplemented as shown in Table 2 before addition to the fibrinogen component. As will be clear to one of skill in the art, medium not intended for use in a CO₂-enriched atmosphere (commonly used in tissue culture incubators or sealed flasks) must be appropriately buffered by some other system. Such media, supplemented with, for instance, HEPES, are well-known in the art. Liebowitz L-15 medium relies on a phosphate buffering system. The medium was supplemented with sodium bicarbonate and dextrose, as shown.

For convenience and consistency a standard 'working cell suspension' of 1.5×10^6 cells ml⁻¹ is generally prepared.

Preparation of fibrin sealant

As outlined in Figure 1 and summarised below, Tisseel™ thrombin powder is reconstituted in a calcium chloride solution according to the manufacturer's directions.

Once dissolved, the Thrombin/CaCl₂ solution is further diluted with supplemented L-15 medium to obtain a 'Working Thrombin Solution' and refrigerated until further use for a minimum of 15 minutes. (Gels may also be manufactured with 'Working Thrombin Solution' at room temperature.) Freeze-dried fibrinogen is reconstituted with an aprotinin solution before being added to the working cell suspension in supplemented L-15 medium. Once reconstituted, the fibrinogen should be used within 4 hours, ideally within 1-2 hours.

Working thrombin solution (6.75 ml) contains:

Thrombin: 50IU/ml (or 337.5IU total)

Calcium chloride: 8µmoles/ml (or 54 µmoles total)

In supplemented L-15

(Total refers to the amount in 6.75mls)

Working fibrinogen and cell suspension mix (total volume 6.75ml):

Tisseel: 19mg/ml (or 128.25mg total)

Aprotinin: 600KIU/ml (or 4050KIU total)

Cells: 1.2x10⁶ cell/ml (8.1x10⁶ cells total for P-1500);

0.4x10⁶ cell/ml (2.7x10⁶ cells total for P-500)

In supplemented L-15

(Total refers to the amount in 6.75mls)

Table 2 Details of Medium Used in the Invention

| Components | Function | Concentration per ml |
|-------------|---|----------------------|
| L-15 medium | Nutrient delivery to the cellular component of the product. Maintains | N/A (base medium) |

| | | |
|--------------------------------------|--|---------|
| | cell viability and structure of the gel. | |
| Sodium Bicarbonate | Required for cell viability | 202.5µg |
| Dextrose | Nutrient | 4.5mg |
| Adenine | Base required for cell viability | 24.4µg |
| L-Glutamine | Amino acid for cell viability | 0.29mg |
| Ethanolamine | Phospholipid for cell metabolism | 6.2µg |
| O-phosphoryl-ethanolamine | Phospholipid for cell metabolism | 14.12µg |
| Hydrocortisone | Steroid required for cell metabolism | 0.4mg |
| Human Recombinant Insulin | Essential hormone | 5µg |
| Selenious acid | Trace substrate for metabolism | 6.78ng |
| 3,3',5-Triiodo-L-thyronine | Hormone | 1.35ng |
| apo-Transferrin, bovine | Cofactor for iron metabolism | 5µg |
| Gamma Irradiated Foetal Bovine serum | Nutrients | 2%v/v |

Casting the gels

The working thrombin solution (6.75ml) and Tisseel™ fibrinogen/cell suspension mixture (6.75ml) are combined by means of a Duplojet mixer unit and loaded into a suitable pre-coated casting container (conveniently a sterile Petri dish or similar) via a 16G needle or equivalent. It is necessary to pre-coat the casting dish with serum containing media or albumin to prevent the gel from adhering. The gel sets within a few minutes. The gel is then bathed in 20ml of medium (Table 2) and the casting dish covered with a lid. The set gel incubated at 37°C for 16–24 hours to allow maturation of the cells.

Packing and Storage

After maturation, the set gels are removed from their casting containers and placed into pre-irradiated, sterile foil pouches, stored within a sterile roto-seal bag. 10ml serum-free medium (as per Table 2, without the foetal bovine serum) is added to each pouch before sealing. The shelf life of the sealed units is up to 28 days at 4°C.

Example 2 Low protein concentration product

For certain applications, it is possible to use lower protein concentrations. The chief advantage of this is reduction of production costs, since serum-derived proteins and many protease inhibitors, such as aprotinin, are expensive. In a preferred embodiment, the concentration of fibrin in the set product is reduced to less than 7 mg ml⁻¹. In practice, 3.0–4.0 mg ml⁻¹ is found to be effective.

One important consideration is the effectiveness (as well as the cost) of aprotinin as the protease inhibitor in such 'low protein' products. In particular, *pro rata* dilution of commercial products results in aprotinin concentrations that are too low to be effective. A preferable solution is to use an alternative inhibitor, such as tranexamic acid. Not only is this a highly effective inhibitor of fibrinolysis, but has significant cost advantages.

Matrix

In this embodiment the matrix protein is fibrin, sourced from a commercial fibrin sealant, TisseelTM using tranexamic acid instead of aprotinin. The key components of the matrix are summarised in Table 3. It should be noted that the same matrix composition could also be achieved using another commercially available fibrin sealant, Quixil. However the addition of exogenous tranexamic acid should be reduced as it already contains this inhibitor.

Table 3 Components of the Fibrinogen Matrix

| Component | Final concentration in cellularised scaffolds |
|-----------|---|
|-----------|---|

| | |
|-----------------------------|----------|
| Matrix protein (fibrinogen) | 3.5mg/ml |
| Tranexamic acid | 10mg/ml |
| Thrombin | 25IU/ml |
| Calcium chloride | 4mM |

Freeze-dried Tisseel™ fibrinogen is reconstituted with supplemented L-15 medium solution before being added to the working cell suspension in supplemented L-15 medium. Once reconstituted, Tisseel™ fibrinogen should be used within 4 hours, ideally within 1-2 hours.

Tisseel™ thrombin powder is reconstituted in a calcium chloride solution according to the manufacturer's directions. Once dissolved, the thrombin/CaCl₂ solution is further diluted with supplemented L-15 medium containing tranexamic acid to obtain a working thrombin solution.

The cell density used is again in the range 450–2500 cells mm⁻². In order to minimise costs, it may be desirable to use a cell density of approximately 450–550 cells mm⁻². It should be noted, however, that protein concentration and cells density are independent variables. Lowering protein concentration is the major cost determinant, rather than cell density. However, being able to use fewer cells may have implications for the speed of production. In any case, high cell density / low protein concentration and low cell density / high protein concentration embodiments are envisaged and may be preferred in specific circumstances.

Example 3 Packaging, storage and delivery

A major factor contributing to the success of topical wound healing compositions is the ease of accurately applying them to the wound surface so that a close contact is established, without air bubbles or creases, under sterile operating conditions. Such compositions are, by their nature, fragile, and handling should be kept to a minimum. The composition of the invention is packaged in such a way as to significantly assist and facilitate application. In addition, the composition is shipped and stored chilled, rather than frozen, so that detailed thawing procedures are not required prior to use.

After setting and the 16–24 hour culture and maturation period, the individual gel discs are packaged by insertion into a flexible foil or metallised plastic pouch comprising two rectangular sheets, sealed along a substantial portion of three of their sides so as to form an open pocket. The inner surface of one of these sheets is modified so as to increase its adherence to the gel product. In a preferred embodiment the packaging used is an Oliver Products (Grand Rapids, Michigan USA) peelable foil pouch comprising one foil sheet and one sheet of laminated polyester/foil sheet with Q15 Adhesive dot pattern coating. Q15/48BF1 is a laminated lidding and pouching material for medical devices. The purpose of this dot pattern adhesive coating is to improve the efficiency of the heat sealing process which is used to seal the edges of the sheets together. However, the adhesive and raised dot pattern prove highly effective in providing a surface to which composition preferentially adheres, as compared with the smooth, uncoated inner surface of the opposing sheet. It will be clear to one skilled in the art that other forms of coating and/or roughening of the surface of one of the internal surfaces of the pouch could be used to achieve the same effect. Similarly, any suitably durable, flexible, water and gas-impermeable sheet material might be used to manufacture such a pouch. All or part of the packaging might be transparent to allow visual inspection, for example, of the integrity of the composition or of the colour of a pH indicator dye in the cell culture medium, a small volume of which is inserted in the pouch, along with the composition, before the pouch is sealed along its remaining open edge.

Thus sealed, the composition has a shelf-life of at least 7–11 days, and preferably up to 28 days, more preferably 21 days, at 2°–8°C.

For application, the pouch is peeled apart, under sterile conditions, leaving the composition adhering to the treated inner surface of one of the sheets comprising the pouch. Using the sheet as a backing or means of support the composition is then applied to the surface of the wound, to which, in the absence of excessive exudation, it will preferentially adhere allowing it to be peeled away from the sheet. This means of application allows the composition to be applied without wrinkling or incorporation of air bubbles,

and with the minimum of manipulation. The edges of the composition may be easily trimmed to fit the limits of the wound. Another advantage of delivering the composition in a format that is reversibly adherent to the packaging, as described, is that it allows the easy identification of the orientation of the product and facilitates oriented application, should this be required. In the case of a homogenous wound-healing product, it is unimportant which way up it is applied to the wound. However, where a multilayered composition is involved, such as one with a fibroblast layer that is intended to be applied in contact with the wound surface and a keratinocyte layer that is intended to be oriented away from the wound surface, it may be difficult or impossible to establish the orientation visually. Then the ability to deliver the product in such a way as makes incorrect application impossible without first removing the composition from the packaging offers a significant advantage.

Example 4

Materials and Methods

By way of example, Human Dermal Fibroblasts (HDFs) of the same passage number and origin were seeded into matrix, as specified below, or seeded without exogenous matrix into tissue culture-appropriate containers, after a 24 hour period of maturation which began on day 0, they were examined to profile gene expression in the constructs:

- i HDFs + Collagen
- ii HDFs + Fibrin
- iii HDFs (no exogenous matrix applied)
- iv Product manufactured to production specifications.

Constructs *i*, *ii* and *iii* were examined on day 1, 5, 14, and 21 each in duplicate. Construct *iv* was examined 9 days after placement into the sealed, sterile package and storage as described, above.

Semi-Quantitative PCR

Total RNA was extracted from all constructs using TriReagent (Sigma) following the manufacturers protocol for total RNA extraction. The integrity of

RNA was tested by electrophoresis of a sample of the isolated RNA through a 2% agarose (InVitrogen) gel and staining with ethidium bromide (Sigma) to visualise the RNA in the gel. In order to analyse gene expression an aliquot of the isolated RNA (10^{-4} -1500 ng RNA) was subjected to reverse transcription and PCR amplification as described in Brady & Iscove (1993, Methods Enzymol. 225: 611-623) using the primer Not1dT (5'CAT CTC GAG CGG CCG CTT TTT TTT TTT TTT TTT TTT T 3'; SEQ ID NO: 1) to produce polyA cDNA. The relative quantities of the resulting products were estimated by electrophoresis of a small sample (1µl) of the PCR products (polyA cDNA) through a 2% agarose gel using dilutions of a known concentration of maximally sheared (100-800 bp) diploid human genomic DNA as standards. Using electronic gel documentation (Syngene), the amount of polyA cDNA representing each RNA sample, was estimated relative to the genomic standard and this information was further used to normalise gene expression data yielded by TaqMan® Realtime/Q-PCR analyses (see below).

PolyA cDNA products were diluted (typically 1000-fold) and aliquots from each sample processed for TaqMan® quantitative PCR using primers to specific genes to amplify corresponding template, if any was present, in the polyA cDNA collections. In order to detect specific amplification products resulting from amplification in TaqMan® real-time PCR, synthesised products were labelled fluorescently by inclusion of SYBR-Green (Molecular Probes) in the reaction mixture. As cDNA is synthesised in the real-time PCR reaction, the fluorescent signal is incorporated into the PCR product and is detected by the instrument (ABI 7700 or equivalent, Applied Biosystems Inc.). The amount of fluorescent signal is directly proportional to the amount of starting template in the reaction. The point at which the fluorescent signal is detectable by the instrument is called the "threshold cycle" and is called the Ct value.

The standard TaqMan reaction is 40 cycles as indicated in the manufacturer's instructions. The first cycle at which SYBR-green labelled PCR product can be detected by the instrument is called the "threshold" cycle (Ct) for the gene under investigation. If there were no template to amplify, no fluorescence

would be incorporated since no product is made, and the Ct value would be 40 (i.e. fluorescence was not detectable after 40 cycles). In a standard reaction of 40 cycles, a reaction that produced no fluorescent signal upon completion of 40 cycles would be equivalent to a product in which no template for amplification had been included. A Ct value less than 40 indicates that the primers recognised a target template and that DNA was synthesised incorporating the fluorescent SYBR-Green "tag".

Table 3. PCR primers for amplification of specific human genes by TaqMan Real-time PCR

| Primer Abbreviation | Gene Name/Designation | 5' to 3' sequence |
|---------------------|--|-------------------------------|
| HsAPOD_54F | Apolipoprotein D | GGTAACAGGGTAGGGCATGGT |
| HsAPOD_136R | | CCACCCCCCCCCATAAA |
| HsMMP2_520F | Matrix metalloprotease 2 | GGGCTGAGCGGGAAGC |
| HsMMP2_606R | | CCCCTGTTCACTCTACTTAGCATGT |
| HsCol3A1_299F | Collagen 3a1 | CATTAGCACCATAACATGCGTCTT |
| HsCol3A1_382R | | GGTGCTCCTCTTTTTCTTGTC |
| HsGas6_103F | Growth arrest specific 6 | GGGCCCACGGCTGAGT |
| HsGas6_173R | | GGCCTGTAACATATCTGTAAATAGTGAGA |
| HsPAI_70F | Plasminogen activator inhibitor | GCACTCAAGGGCAAGGATATG |
| HsPAI_150R | | GCGTGCCCAGCTCTTCAC |
| HsPLAU_196F | Urokinase | AAACTGAGACAGTGCTGGTCACA |
| HsPLAU_268R | | GGGTCCCCACGTGACA |
| HsVIM_174F | Vimentin | TTGTAGGAGTGTCGGTTGTTAAGAAC |
| HsVIM_261R | | TCAAGTGCCTTCTGCAGTTTTT |
| HsSMAActin_194F | Smooth muscle actin | GGCCCGGCTTCATCGTAT |
| HsSMAActin_267R | | GGCTCCATCCTGGCCTCT |
| HsPDGFb_109F | Platelet derived growth factor | CCCCAAAAATATAATCACCGACTT |
| HsPDGFb_200R | | CACCTCCCTTCCCACCTACTG |
| HsCOX2_154F | Cyclo-oxygenase 2 | AAACGAAGTGTTTGAGAAGACTGTGT |
| HsCOX2_262R | | AATTCAGTAGGTGCATTGGAATCA |
| HsGAPDH_F | Glyceraldehyde 3-phosphate dehydrogenase | ACACTCAGACCCCCACCACA |
| HsGAPDH_R | | CATAGGCCCTCCCCTCTT |
| HsRPL32_402T | Ribosomal protein L32 | CTGGCCATCAGAGTCACCAA |
| HsRPL32_466R | | TGAGCTGCCTACTCATTTTCTTCA |
| HsCol6A1_276F | Collagen 6a | CACCGTTAATCTCGAGGGTCTT |
| HsCol6A1_342R | | TGACCCCGACCTCAGAGAGTAC |
| HsINSIG1_194F | Insulin induced gene 1 | AATGAAATCGAATACTTGGGAAGCT |

| | | |
|---------------|---|---------------------------|
| HsINSIG1_268R | | TCTGTGCCCTGGAGCATTCT |
| HsCol1A1_231F | Collagen 1a1 | GGATGGAGGGAGTTTACAGGAA |
| HsCol1A1_296R | | GTGCCCCAGACCAGGAATT |
| DD2_42F | gi 7022020 | TCCCTGTGCCCAGAGTAACC |
| DD2_114R | | AGGTCTGGCTCCTGTGTTTTACA |
| DD4_386F | gi 46267369 | TTATTGAAAGCTGACCTGCTAATGA |
| DD4_459R | | GGGCAGTCACCCATTCAATT |
| DD5_116F | X-ray repair, complementing defective, in chinese hamster, 1 (XRCC1) | CCCATAGAGCTGGTGAGGAAGT |
| DD5_182R | | CGTTCGTCCCCGATGGA |
| DD10_98F | gi 10437022 | GTCCACAGTGCCCCTTCCT |
| DD10_169R | | CGGTCCCTGGCATCATG |
| DD12_154F | Ribosomal protein S8 | AAGCGATGCACGCAAGAAG |
| DD12_223R | | AAGAATGCCAAAATCAGCAGTCT |
| DD13_77F | Huntingtin Interacting Protein K | GGCAATAAGCGCCTCTACCA |
| DD13_142R | | CCTCGAGCAGCAGCAGAAC |
| GB1_74F | gi 24810897 | TCAGGGCAACACCACACACT |
| GB1_156R | | CCATGTTTGAGCTTCTGTTTCAA |
| GB5_278F | Ribosomal protein S24 | TCATGCCAAAGCCAGTTGTC |
| GB5_351R | | CACACCGGATGTCATCTTTGTATT |

Differential Display

In order to quickly visualise and, by way of example, identify differences in gene expression between samples cast in different or no matrix, an aliquot of each RNA sample was processed by conversion to polyA cDNA products for analysis by differential display techniques. Briefly, polyA cDNA products were amplified using a collection of proprietary primers (Epistem Ltd. UK) designed to prime DNA synthesis at random and reveal differences in gene expression between two samples. In the illustration shown in Figure 1a, two primers A and B indicated by broken grey lines, have annealed to homologous sequences on a single cDNA molecule, representing gene Y, to prime synthesis of a PCR product, AB, of relatively small size. In the illustration, the arrows indicate the direction of DNA synthesis from the primers. Conversely, in Figure 1b, a second pair of primers have annealed to a second cDNA, representing gene Z, at sites C and D at relatively greater distance from each other, resulting in a PCR product, CD, of relatively larger size. Electrophoresis reveals the PCR products AB and CD as bands migrating through the

electrophoresis medium closer or farther from the migration origin of the medium (i.e. the top of the gel).

In Figure 1c, cDNA products AB and CD from three different samples, 1,2,3, are illustrated. In sample 1, electrophoresis reveals product AB as a more intense band (rel. high abundance) that has moved to the bottom of the gel (small product size) whereas in sample 2, the same product yields a band of identical size but of relatively less intensity, signifying that more of product AB was present in sample 1 than in sample 2. Similarly, product CD, migrating proximal to the origin of electrophoresis (larger size) produces a relatively more intense band in samples 1 and 2 than in sample 3, indicating its relative paucity in sample 3 compared to samples 1 and 2. Products sharing the same position after electrophoresis may represent a single identical product expressed in all samples whilst those present in only some samples may be expressed specifically under the conditions present when that sample was collected. PolyA cDNA products from different samples will thus produce a characteristic pattern of large/small, abundant/rare, products similar to a "bar code" for that sample (Figure 2).

Some of these noted differences in the patterns of gene expression were further processed, by proprietary DNA sequencing techniques (Epistem Ltd, UK) to identify the genes that these bands represent.

Table 4.

| Age Cold | Young Profile (d1-d5) | Older Young Profile (d1-d14) | Other |
|---------------------------|--------------------------------|--|---|
| Shipping Profile (eg 4°C) | Box 2 √DD5 √DD10 √GB1 | Box 1 √ApoD √MMP2 √Col3A1 √SMA | Box 3 √GB5 √Gas6 √GAPDH √PAI √DD2 √DD12 √PLAU √Vimentin |
| Other (eg 37°C) | Box 5 √GB5 √DD12 | Box 4 √DD2 | Box 6 √RPL32 |

The effects of maturation/manipulation over time and of storage/shipping conditions on gene expression in the products of the invention are summarised in Table 4. HDFs cast in fibrin, collagen, or cast onto tissue-culture appropriate plastic, were analysed for gene expression over 21 (31) days after casting (Age). In addition, HDFs cast in fibrin were stored at 4°C ($\pm 4^\circ\text{C}$) and assayed over a period of 22 (30) days (Cold).

Several overlapping profiles emerged from this analysis:

- 1/ Genes expressed in young cells subjected to a minimum of further manipulation after casting (up to 14 days) at 37°C and expressed under storage conditions (Box 1)
- 2/ Genes expressed in young cells subjected to no further manipulation after casting (up to 5 days) at 37°C and expressed under storage conditions (Box 2)
- 3/ Genes expressed specifically under storage conditions (Box 3)
- 4/ Genes expressed specifically in young cells subjected to a minimum of further manipulation after casting (up to 14 days) at 37°C (Box 4)
- 5/ Genes expressed specifically in young cells subjected to no further manipulation after casting (up to 5 days) at 37°C (Box 5)
- 6/ Genes expressed non-specifically under all circumstances investigated (Box 6).

Table 5 Ranges of fold change in gene expression compared to RPL32

| Gene acronym | Gene Name or accession number | range | Preferred range | old |
|---------------|---------------------------------|---------------------------|-----------------|----------|
| APOD | Apolipoprotein D | 2-48,000 | 100-2,000 | 15,000 |
| MMP2 | Matrix metalloprotease 2 | 2,000-1.6x10 ⁶ | 13,000-100,000 | 290,000 |
| Col3a1 | Collagen 3a1 | 20-44,000 | 800-1,900 | 23,000 |
| Gas6 | Growth arrest specific 6 | | | |
| PAI | Plasminogen activator inhibitor | | | |
| PLAU | Urokinase | | 130-760 | 1400 |
| Vim | Vimentin | | 28,000-2,065,00 | 4,800,00 |

| | | | | |
|----------------|--|---------------|---------------------------------|------------|
| SMA | Smooth muscle actin | 20-150,000 | 1,600-8,600 | 22,000 |
| PDGF | Platelet derived growth factor beta | | | |
| Cox2 | Cyclo-oxygenase 2 | | | |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase | | | |
| Coll6a | Collagen 6a | | | |
| INSIG1 | Insulin induced gene 1 | | | |
| Coll1a1 | Collagen 1a1 | | | |
| DD2 | gi 7022020 | 0-750,000 | 0-4 (0-13d) 0-136,000(+cold) | |
| DD4 | gi 46267369 | | 0.3-1.6 | 5.00 |
| DD5 | X-ray repair, complementing defective, in chinese hamster, 1 | 0.6-500 | 13-160 | 560.00 |
| DD10 | gi 10437022 | 0-210 | 3-15 | 94.00 |
| DD12 | Ribosomal protein S8 | 120-36,000 | 1,000-30,000 | |
| GB1 | gi 24810897 | 0-33 | 0-5 | 36.00 |
| GB5 | Ribosomal protein S24 | 1,000-120,000 | 11,000-53,000 | 100,000.00 |

Claims

1 A wound healing composition comprising isolated living mammalian cells having a wound healing phenotype, wherein the cells:

(a) have been incubated in a protein rich environment for approximately 0-96 hours; and/or

(b) exhibit 2 to 48000, more preferably 100 to 2000, fold increase in expression of ApoD; 2000 to 1600000, more preferably 13000 to 100000 fold increase in expression of MMP2; 20 to 44000, more preferably 800 to 1800 fold increase in expression of Col3a1; and/(or) 20 to 150000, more preferably 1600 to 2500 fold increase in expression of SMA, as compared to the expression of RPL32; and/or

(c) have a banding pattern of PCR products resulting from differential display, similar to that of the nucleic acid expression in fibrin as shown in Figure 4 or Figure 5.

2. A wound healing composition according to claim 1, wherein the protein rich environment of (a) comprises fibrin collagen, fibronectin, vitronectin, alginate, agar, hyaluronic acid, modified starches, carrageenans, carob, gelatine, pectin, gelling agents.

3. A wound healing composition according to claim 1 or 2 wherein the cells of (a) are incubated for 0 – 96 hours, 0 – 48 hours, 0 – 25 hours or 16 – 24 hours, during which they reach a wound healing phenotype.

4. A wound healing composition according to claims 1 to 3 wherein the fold increase in expression levels for ApoD are 100 to 2000.

5. A wound healing composition according to claims 1 to 4 wherein the fold increase in expression levels for MMP2 are 13000 to 1000000.

6. A wound healing composition according to claims 1 to 5 wherein the fold increase in expression levels for Col3a1 are 800 to 1800.

7. A wound healing composition according to claims 1 to 6 wherein the fold increase in expression levels for SMA are 1600 to 2500.

8. A wound healing composition according to any of the previous claims wherein the cells of (b) also exhibit 0 to 500 fold increase in expression of DD5, 0 to 210 fold increase in expression of DD10 and/or 0 to 33 fold increase in expression of GB1 as compared to RPL32 during days 0 to 4.

9. A wound healing composition according to any of the previous claims wherein the cells of (b) also exhibit 1000 to 120000 fold increase in expression of GB5; 120 to 36000 fold increase in expression of DD12 and/or 0 to 750000 fold increase in expression of DD2 as compared to RPL32 during days 0 to 4.

10. A wound healing composition according to any one of claims 1 to 9 wherein the mammalian cells are fibroblasts, preferably dermal fibroblasts.

11. A wound healing composition according to any one of claims 1 to 10 wherein the cells are human.

12. A wound healing composition according to any one of claims 1 to 11 wherein the cells are actively synthetic or able to rapidly become actively synthetic.

13. A wound healing composition according to any one of claims 1 to 12 wherein the cells are not proliferating or senescent.

14. A wound healing composition according to any one of claims 1 to 13 wherein the cells are provided in a medium or a biocompatible matrix.

15. A wound healing composition according to claim 15 wherein the matrix is protein based, and preferably is a clottable or gelling substance, preferably fibrin collagen, fibronectin, vitronectin, alginate, agar, hyaluronic acid, modified starches, carrageenans, carob, gelatine, pectin, gelling agents.

16. A wound healing compositions according to claim 15 wherein the matrix is fibrin gel.

17. A wound healing composition according to any one of claims 14 to 16 wherein the matrix is sterile and non-pyrogenic.

18. A wound healing composition according to any one of claims 14 to 17 wherein the cells are cast in the matrix prior to topical application.

19. A wound healing composition comprising living mammalian cells within a sterile, non-pyrogenic matrix characterised in that said matrix is pre-cast before topical application.

20. A wound healing composition according to any one of claims 14 to 19 wherein said matrix is approximately 8mm or less thick, preferably 5mm or less.

21. A wound healing composition according to any one of claims 14 to 20 containing approximately 450 to 2500 cells per mm².

22. A wound healing composition according to any one of claims 14 to 21 containing approximately 450 to 550 cells per mm².

23. A wound healing composition according to any one of claims 14 to 22 containing approximately 900 to 1700 cells per mm².

24. A wound healing composition according to any one of claims 14 to 23 concentration of protein in the matrix is in the range 3-12mg ml⁻¹.

25. A wound healing composition of claim 24 wherein the concentration of fibrin is in the range 3–5mg ml⁻¹.
26. A wound healing composition of claim 24 wherein the concentration of fibrin is in the range 7–12mg ml⁻¹.
27. A wound healing composition according to any of claims 14 to 26 for use as a medicament.
28. A wound healing composition according to any of claims 14 to 26 for use as a medicament in the treatment of a skin lesion.
29. A wound healing composition of claim 28 wherein said skin lesion is a venous ulcer, diabetic ulcer, pressure sore, burn or iatrogenic grafting wound.
30. The use of living mammalian cells as defined in any one of claims 1 to 14 in the manufacture of a wound healing composition for the treatment of a skin lesion.
31. The use of a wound healing composition according to claim 30 wherein the skin lesion is a venous ulcer, diabetic ulcer, pressure sore, burn or iatrogenic grating wound.
32. Isolated living mammalian cells as defined in any one of claims 1 to 14.
33. A method of manufacturing a wound healing composition comprising: suspending living mammalian cells in a solution comprising a protein monomer capable of polymerisation into an insoluble matrix, adding an agent capable of promoting such polymerisation and allowing polymerisation to occur in a mould such that the solid polymerised composition may be removed and packaged ready for topical administration to a patient.
34. The method of claim 33 wherein said monomer is fibrinogen.

35. The method of claims 33 or 34 wherein said agent capable of promoting polymerisation is thrombin.

36. The method of any of claims 33 to 35 wherein said cells are fibroblasts.

37. The method of claim 36 wherein said fibroblasts are human dermal fibroblasts.

38. The method of any one of claims 33 to 37 wherein the composition contains 450 to 2500 cells per mm^2 .

39. The method of claim 38 wherein the composition contains 450 to 550 cells per mm^2 .

40. The method of claim 38 wherein the composition contains 900 to 1700 cells per mm^2 .

41. The method of any of claims 33 to 40 wherein the concentration of fibrin in the solid polymerised composition is in the range of 3 – 12 mg ml^{-1} .

42. The method of claim 41 wherein the concentration of fibrin is in the range 3 – 5 mg ml^{-1} .

43. The method of claim 41 wherein the concentration of fibrin is in the range 7 – 12 mg ml^{-1} .

44. The method of any of claims 33 to 44 further comprising use of a protease inhibitor.

45. The method of claim 44 wherein said protease inhibitor is selected from the list consisting of aprotinin and tranexamic acid.

46. The method of claim 45 wherein the protease inhibitor is tranexamic acid.

47. The method of any one of claims 33 to 46 for the manufacture of a wound healing composition according to any one of claims 1 to 31.

48. Use of living mammalian cells in the manufacture of a wound healing composition by the method of any one of claims 33 to 46 for the treatment of a skin lesion.

49. The use of claim 48 wherein the skin lesion is a venous ulcer, diabetic ulcer, pressure sore, burn or iatrogenic grafting wound.

49. A method of treating a patient suffering from a skin lesion comprising the topical application of the composition of any one of claims 1 to 30 to said skin lesion.



10

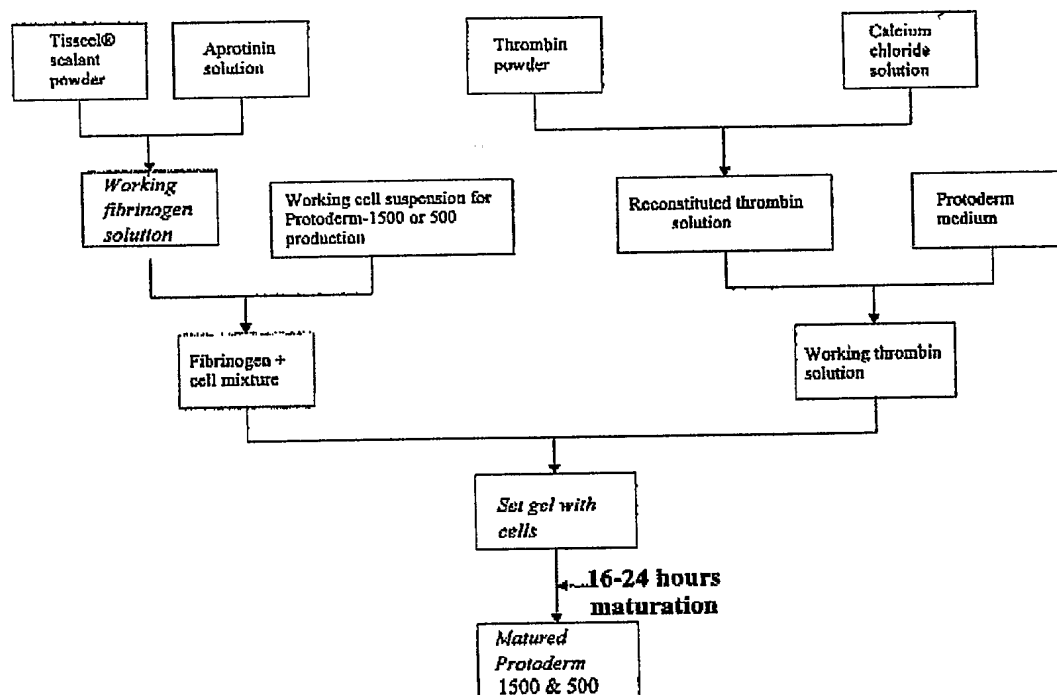
11

12

13

1 of 9

Figure 1





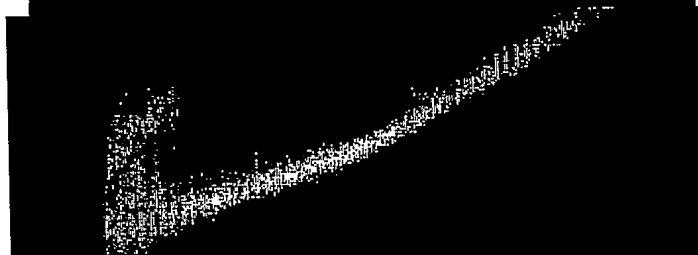
2 of 9

Figure 2

A



B



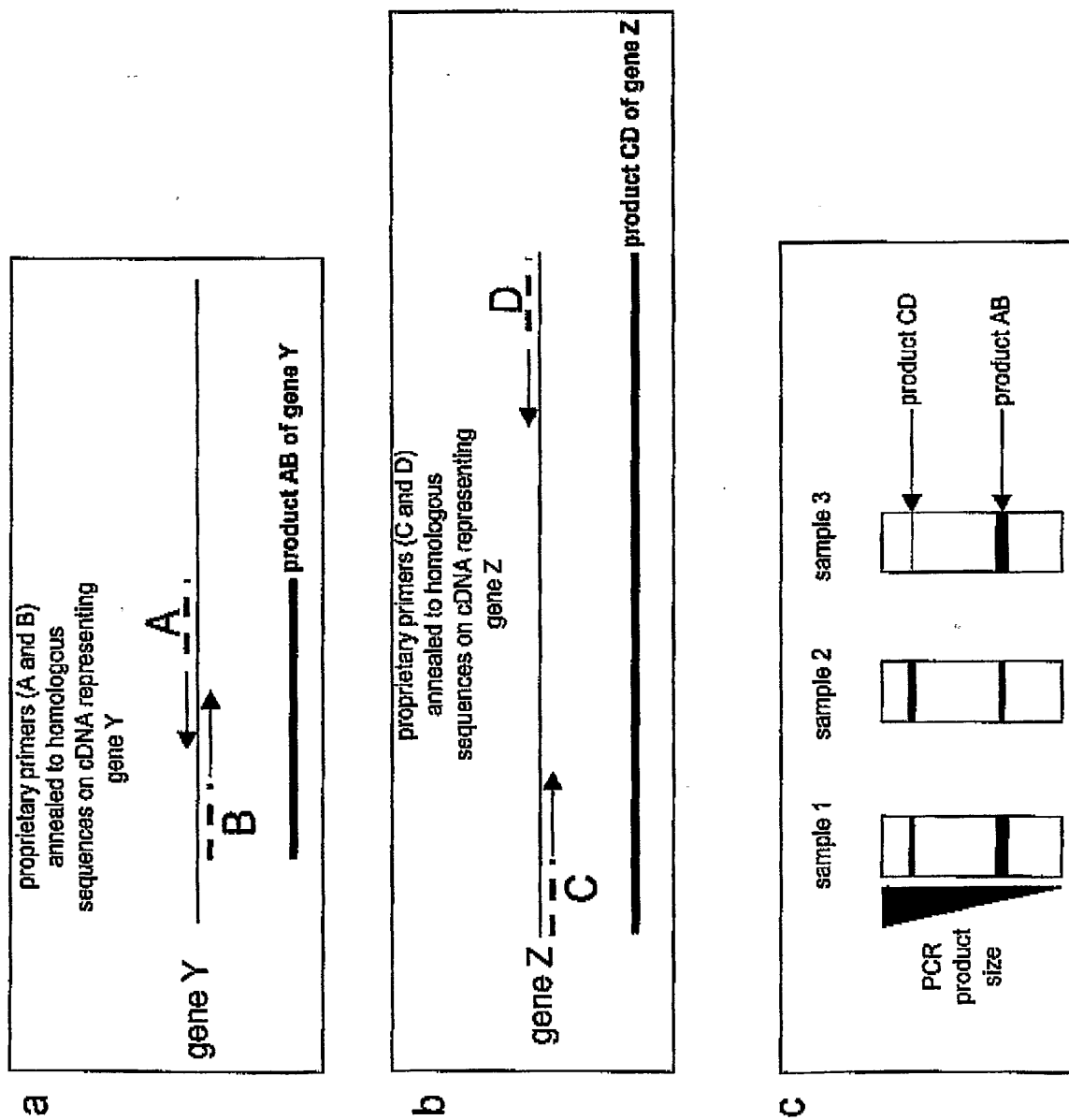
C





3 of 9

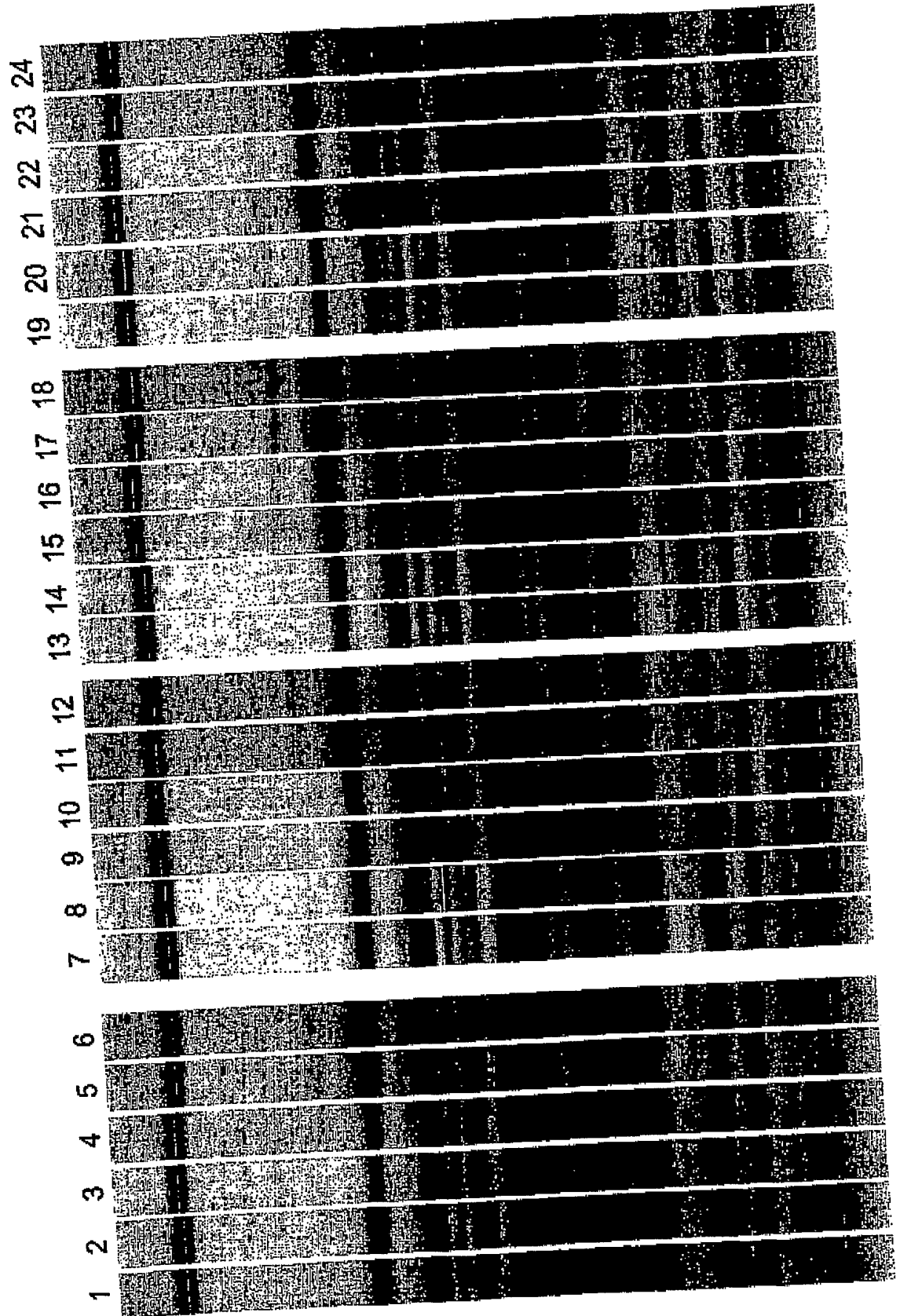
Figure 3.





4 of 9

Figure 4.





5 of 9

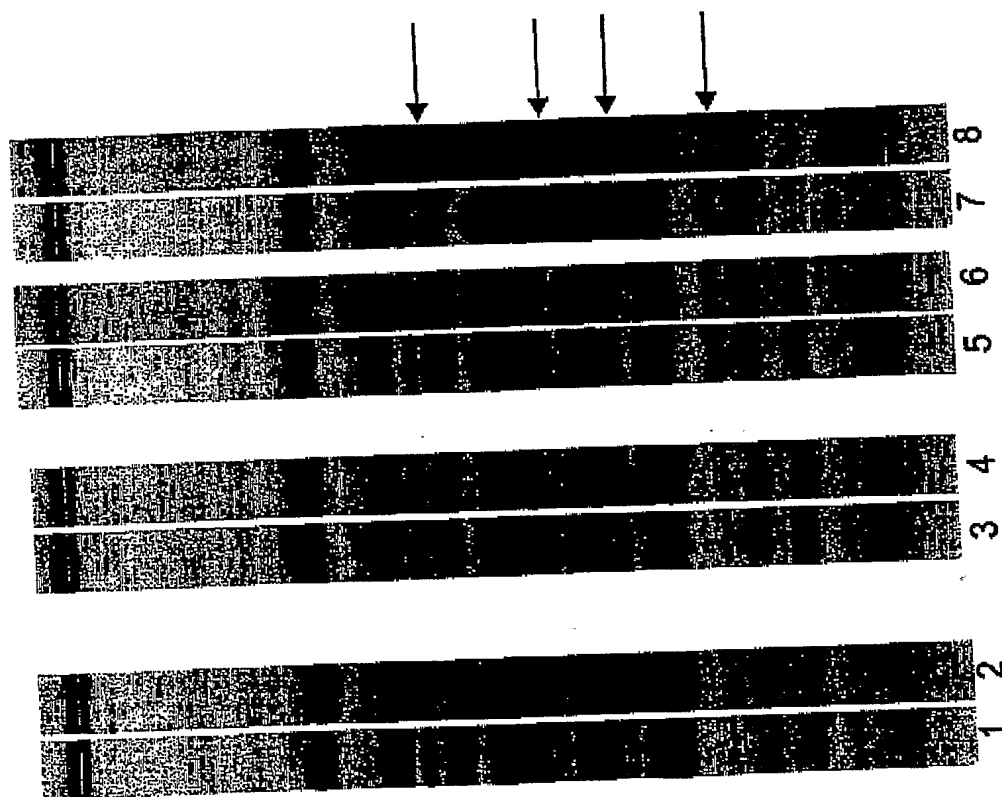


Figure 5.



6 of 9

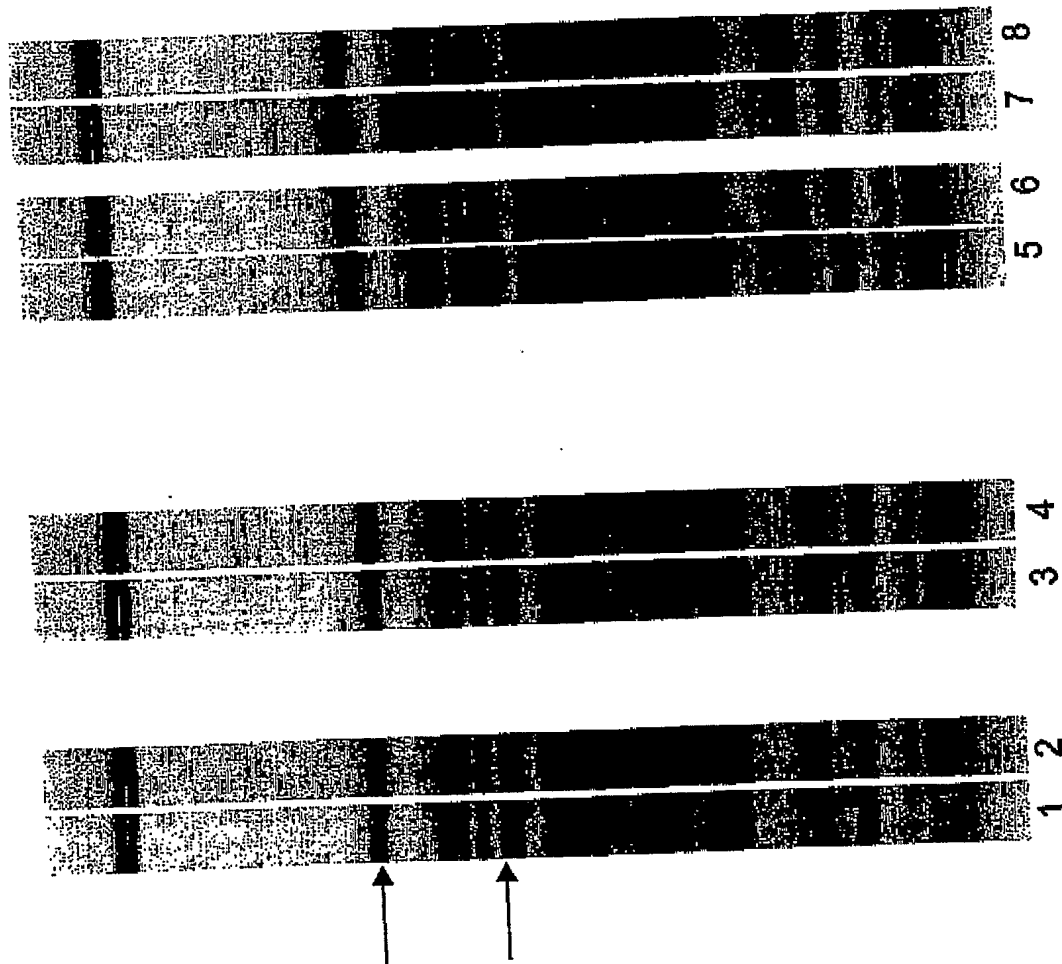
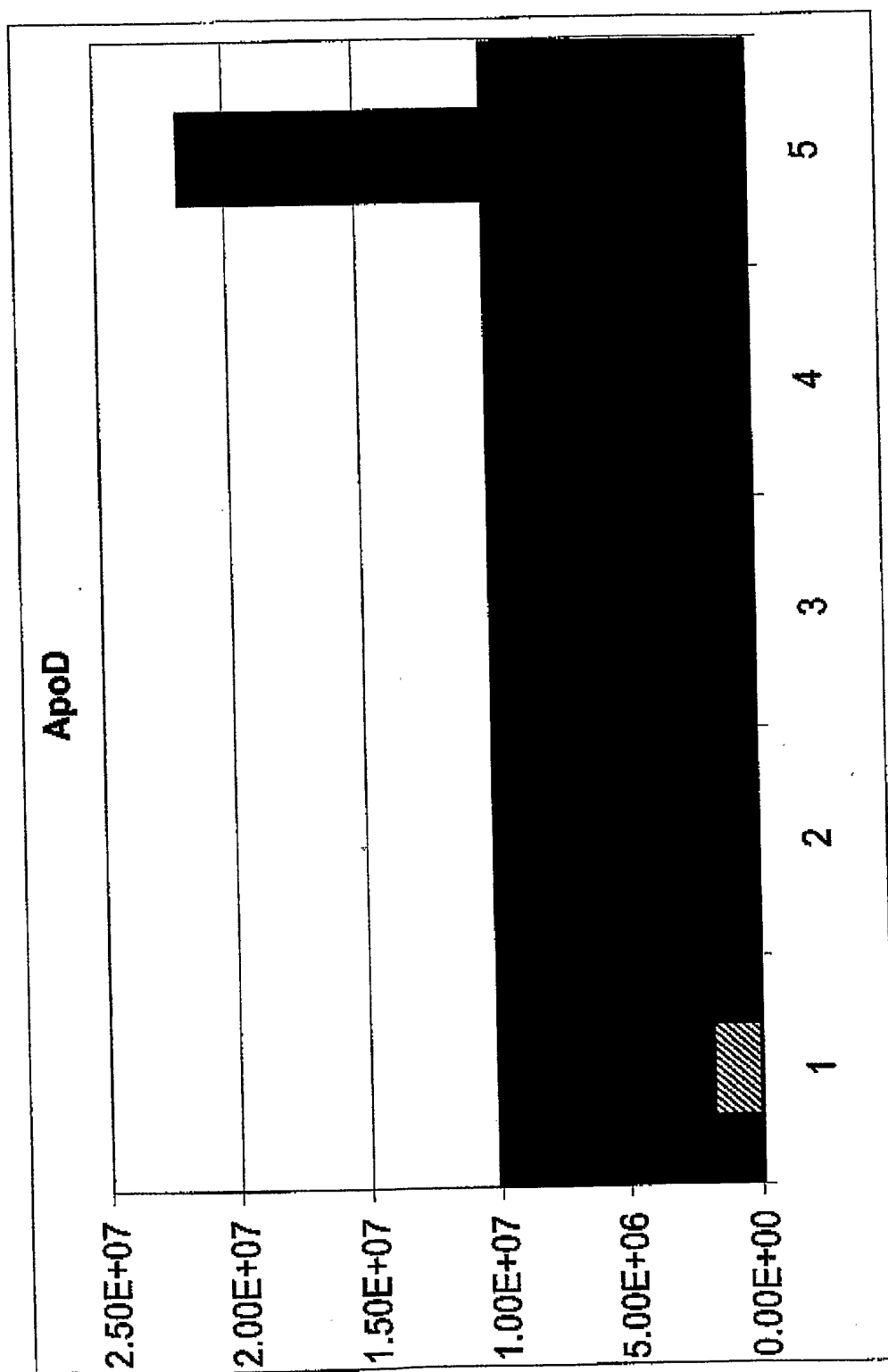


Figure 6.



7 of 9

Figure 7.





8 of 9

Figure 8.

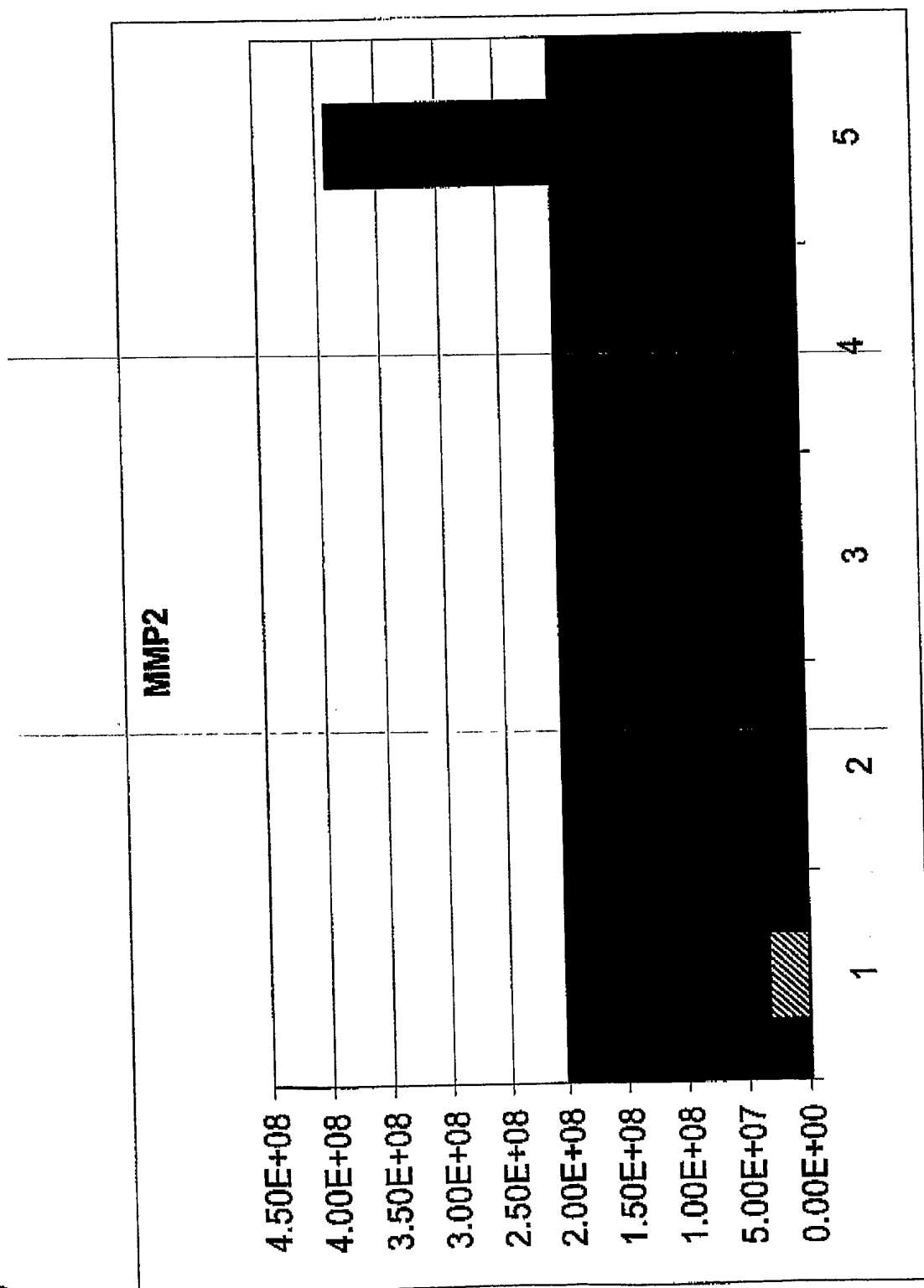
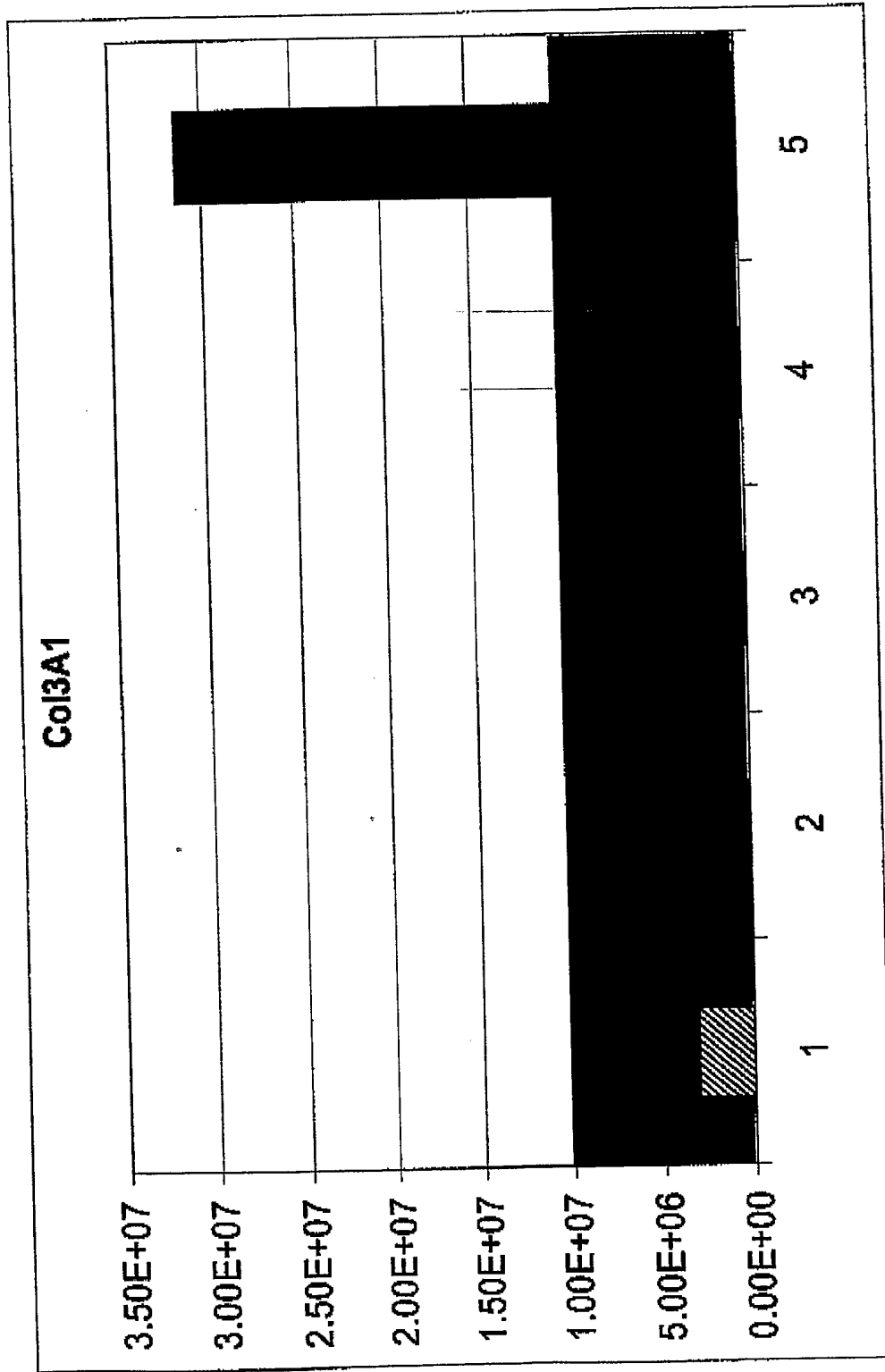




Figure 9.





PCT/GB2005/000523

